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Insecticidal spectrum and mode of action of the *Bacillus thuringiensis* Vip3Ca insecticidal protein

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ABSTRACT

The Vip3Ca protein, discovered in a screening of Spanish collections of *Bacillus thuringiensis*, was known to be toxic to *Chrysodeixis chalcites*, *Mamestra brassicae* and *Trichoplusia ni*. In the present study, its activity has been tested with additional insect species and we found that *Cydia pomonella* is moderately susceptible to this protein. Vip3Ca (of approximately 90 kDa) was processed to an approximately 70 kDa protein when incubated with midgut juice in all tested species. The kinetics of proteolysis correlated with the susceptibility of the insect species to Vip3Ca. The activation was faster to slower in the following order: *M. brassicae* (susceptible), *Spodoptera littoralis* (moderately susceptible), *Agrotis ipsilon* and *Ostrinia nubilalis* (slightly susceptible). Processing Vip3Ca by *O. nubilalis* or *M. brassicae* midgut juice did not significantly changed its toxicity to either insect species, indicating that the low susceptibility of *O. nubilalis* is not due to a problem in the midgut processing of the toxin. *M. brassicae* larvae fed with Vip3Ca showed binding of this toxin to the apical membrane of the midgut epithelial cells. Histopathological inspection showed sloughing of the epithelial cells with further disruption, which suggests that the mode of action of Vip3Ca is similar to that described for Vip3Aa. Biotin-labeled Vip3Ca and Vip3Aa bound specifically to *M. brassicae* brush border membrane vesicles and both toxins competed for binding sites. This result suggests that insects resistant to Vip3A may also be cross-resistant to Vip3C, which has implications for Insect Resistance Management (IRM).

1. Introduction

Bacillus thuringiensis is a gram positive bacterium that during the vegetative growth phase produces proteins (Vip, phospholipases, proteases, chitinases, etc.) which are secreted to the medium, contributing to the pathogenicity of this bacterium against insects (Schnepf et al., 1998). Vip proteins are a class of secreted insecticidal proteins which were first described in 1996 and which share no sequence homology with the Cry proteins (Estruch et al., 1996). To date, more than 100 *vip3* genes have been identified (http://www.btnomenclature.info/). Vip3Aa was the first member of the family of Vip3 proteins ever described and most studies dealing with the insecticidal activity and mode of action Vip3 proteins have been carried out with this protein (Chakroun et al., 2016). The fact that Vip3A proteins differ from the Cry1 and Cry2 proteins in terms of insecticidal spectra and because they target different midgut binding sites (Lee et al., 2006; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014) makes them ideal candidates to be used in combination with Cry proteins in IRM programs.

The mode of action of the Vip3Aa protein resembles that of the Cry proteins in that they are synthesized in the form of protoxins which are processed by proteases in the larva midgut rendering the active toxin (Yu et al., 1997; Lee et al., 2003). The activated toxin binds to specific receptors in the midgut membrane (Lee et al., 2003, 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014). Finally,

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the bound toxin provokes the disruption of the midgut epithelial cells (Yu et al., 1997; Abdelkefi-Mesrati et al., 2011a,b) by the formation of pores in the apical membrane (Lee et al., 2003; Liu et al., 2011).

A screening of Spanish collections of *Bacillus thuringiensis* was undertaken to search for novel members of the Vip3 family. As a result, new *vip3* genes were discovered constituting a novel family which was classified as *vip3Ca* (Palma et al., 2012). All Vip3Ca proteins discovered so far contain 803 amino acids (molecular mass of approximately 90 kDa) and they have insecticidal activity to some lepidopteran species. The aim of the present paper was to explore additional insect species potentially susceptible to Vip3Ca and, at the same time, to study its mode of action in *Mamestra brassicae*, an insect species that have shown susceptibility to this protein.

2. Materials and methods

2.1. Insect colonies

Insects were grown in the insectaries of the Public University of Navarra and of the University of Valencia at 25 ± 2 °C, 60 ± 5% RH, and L16: D8 h. *Sesamia nonagrioides*, *Grapholita molesta*, *Cydia pomonella*, *Mamestra brassicae* and *Pectinophora gossypiella* were reared in artificial diet (Poitout and Bues, 1974; Greene et al., 1976; Guennelon et al., 1981; Eizaguirre and Albajes, 1992; Pérez-Guerrero et al., 2004). *Galleria mellonella* was reared in a honey based diet (10.5% glycerine, 22% honey, 14% cornmeal, 5.5% brewer's yeast, 14% wheat flour, 14% infant cereals, 9.5% milk powder and 10.5% water). *Plutella xylostella* and *Tuta absoluta* were reared on leaves of *Brassica oleracea* var. *capitata* and *Solanum lycopersicum*, respectively. *Ephestia kuehniella* larvae were reared in wheat flour. *Nezara viridula* nymphs and adults were reared in *Phaseolus vulgaris* pods supplemented with peanuts (*Arachis hypogaea*). *Drosophila melanogaster* was reared on laboratory in cornmeal/yeast medium.

2.2. Source and expression of Vip3 proteins

Vip3Aa (NCBI accession No. AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene (Abdelkefi-Mesrati et al., 2009). The Vip3Ad (NCBI accession No. CAI43276) protein was expressed from *E. coli* WK6 carrying the *vip3Ad2* gene (kindly supplied by Bayer CropScience N.V., Ghent, Belgium). Vip3Ca (NCBI accession No. AEE98106) protein was prepared from recombinant *E. coli* WK6 carrying the *vip3Ca2* gene (Palma et al., 2012).

The Vip3Aa protein was expressed following the conditions described by Chakroun et al. (2012). In the case of the Vip3Ca and Vip3Ad, a single colony was inoculated in 4 ml of LB medium containing 100 μg/ml ampicillin and grown overnight at 37 °C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 μg/ml ampicillin was further incubated at 37 °C and 180 rpm. When the OD was in the range value 0.7–0.8, 1 mM IPTG (final concentration) was added for induction. The culture was growth overnight at 37 °C and 200 rpm. Cells were collected at 6000*g* for 15 min at 4 °C and the pellet was weighed and resuspended in 3 ml lysis buffer (PBS, pH 8.0, containing 3 mg/ml lysozyme, 10 μg/ml DNase, and 100 μM PMSF) per gram of pellet. The sample was incubated at 37 °C for 30 min and then kept in the freezer at least 30 min. Then the sample was sonicated on ice applying two 1 min pulses at 70 W and at a constant duty cycle, separated by a 10 s cooling period on ice. Insoluble material was separated by centrifugation at 16,000*g* for 15 min at 4 °C and the soluble cellular fraction sequentially filtered through sterile 0.45 μm and 0.22 μm cellulose acetate filters (GE Healthcare Life Sciences).

2.3. Purification of the Vip3 proteins

Vip3 proteins used for bioassays or proteolysis assays were purified by immobilized metal ion absorption chromatography (IMAC) on a Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+}. The eluted Vip3 protein was collected on PBS containing 0.1 mM EDTA (pH 8.0) to avoid inactivation of the protein. Buffer exchange was performed immediately with storage buffer (20 mM Tris, 150 mM NaCl, pH 9.0) with a PD-10 desalting column (GE Healthcare) to prevent protein precipitation and aggregation. Concentration of the purified protein was estimated with the Bradford method (Bradford, 1976). The purity of the expressed protein was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R-250 (Sigma-Aldrich).

For histopathological studies, the Vip3Aa, Vip3Ad, and Vip3Ca, proteins were purified by isoelectric point precipitation (Chakroun et al., 2012). The pH of the lysate was lowered with acetic acid to pH 5.5 for Vip3Aa, pH 4.95 for Vip3Ad, and pH 5.95 for Vip3Ca. The pellets were recovered by centrifugation at 16,000*g* for 10 min and then resuspended in storage buffer and dialysed against the same buffer overnight. The purified proteins were stored at −20 °C.

For binding assays, Vip3 proteins were purified by isoelectric point precipitation as described above. Solubilized Vip3 proteins were incubated with 1% trypsin (wt/wt) for 2 h at 37 °C. Activated Vip3Ca and Vip3Aa were used as competitors in binding assays. For biotin labeling, Vip3Aa was further purified by anion-exchange chromatography. After overnight dialysis against 20 mM Tris-HCl (pH 9), the Vip3Aa activated protein was purified on a HiTrap Q HP (5-ml bed volume) column equilibrated in the same dialysis buffer, using an ÄKTA explorer 100 chromatography system (GE Healthcare, United Kingdom). Proteins were eluted with a 100 ml linear gradient (0–80%) of 1 M NaCl.

2.4. Insect toxicity assays

Several methodologies were used in the bioassays depending on the insect species tested. For *M. brassicae*, *S. nonagrioides*, *C. pomonella*, and *G. molesta*, bioassays were performed on neonates using surface contamination assays. Surface contamination assays were also employed with L3 larvae for *G. mellonella* and *P. xylostella*. Bioassays with *P. gossypiella* and *E. kuehniella* were conducted with neonates and L3 larvae, respectively, using toxins in diet incorporation assays. Bioassays with *T. absoluta* were performed on L2 larvae in leaf disks. With the homopteran *N. viridula*, bioassays were conducted on N1 using toxins in feeding assays (Palma et al., 2014). With the dipteran *D. melanogaster*, bioassays were conducted on L1 using surface contamination assays.

For a preliminary screening of the activity of the Vip3Ca protein, two concentrations (0.4 and 4 μg/cm^2) were tested when the assay was done on artificial diet in surface contamination assays. In diet incorporation assays, the Vip3Ca protein was tested at a single concentration of 60 μg/g and 100 μg/g, for *P. gossypiella* and *E. kuehniella*, respectively. For the leaf disk and feeding assays the Vip3Ca was tested also at a single concentration in both bioassays (200 mg/ml and 1 mg/ml, respectively). Sixteen larvae were used for each protein concentration and the bioassay was repeated at least twice. Bioassays were conducted at 25 °C, 60 ± 5% RH, and a 16:8 (light/dark) photoperiod. Mortality and effective mortality (dead larvae plus number of larvae that remained in the initial instar) were scored after 21 days for *P. gossypiella*, 7 days for *S. nonagrioides*, *M. brassicae*, *G. molesta*, *C. pomonella* and *G. mellonella*,

4 days for *P. xylostella*, *E. kuehniella* and *D. melanogaster*, and 3 days for *T. absoluta* and *N. viridula*. Determination of the LC_{50} (concentration of protein killing 50% of tested individuals) for Vip3Ca was done for the most susceptible species (*M. brassicae*, *C. pomonella* and *E. kuehniella*) as reported elsewhere (Ruiz de Escudero et al., 2014). The LC_{50} of Vip3Aa for *C. pomonella* was calculated in the same way as for Vip3Ca. The storage buffer was use to dilute Vip3 proteins and as the negative control. Only the bioassays with less than 10% control mortality were considered.

2.5. Midgut juice preparation and Vip3 proteins processing

Midgut juice preparation was obtained from last instar larvae of *M. brassicae*, *Agrotis ipsilon*, *Spodoptera littoralis* and *Ostrinia nubilalis*. Midguts were dissected and cut open to eliminate food clumps. The midgut with the remaining contents was centrifuged at 16,000*g* for 10 min and the supernatant, which constituted the midgut juice, was recovered. The protein concentration was measured with the Bradford method (Bradford, 1976).

For the time course assays, the Vip3Ca protein was mixed with several dilutions of midgut juice in a final volume of 25 µl and incubated at 30 °C. The reaction was stopped at different time intervals by adding the electrophoresis loading buffer and heating at 99 °C for 10 min. Reaction products were separated by 12% SDS-PAGE and the gels stained with Coomassie brilliant blue R-250 (Sigma-Aldrich).

For the insect toxicity assays, Vip3Aa and Vip3Ca were processed with midgut juice from *M. brassicae* and *O. nubilalis*, and with commercial bovine trypsin (Sigma-Aldrich). Both proteins where incubated with midgut juices for 24 h at 30 °C, at the ratio 100:0.36 and 100:16 (protein:midgut juice of *M. brassicae* and *O. nubilalis*, respectively). Trypsin activation was performed as described above.

2.6. Insect toxicity assays of the activated Vip3 proteins with midgut juice from M. brassicae and O. nubilalis and with trypsin

As for *M. brassicae*, bioassays with *O. nubilalis* were performed on neonates using surface contamination assays and mortality was scored after 7 days. *M. brassicae* and *O. nubilalis* larvae (three replicates of 24 larvae) were fed with the native and the activated forms of Vip3Aa and Vip3Ca. Larvae groups received 0.74 µg/cm^2 and 4.5 µg/cm^2 of each protein form, which corresponded to the LC_{30} and an LC_{90} values, respectively, of Vip3Ca for *M. brassicae*. Bioassays were repeated three times.

2.7. Histopathological effects and in vivo binding of Vip3 proteins in M. brassicae

Vip3Aa, Vip3Ad, and Vip3Ca (6 µg each) were used to intoxicate L2/L3 instar larvae of *M. brassicae*. Briefly, after 1 h, 3 h and 6 h of exposure, larvae were cut at the two ends and immediately fixed with 4% paraformaldehyde (PFA) for two days and then transferred to 30% sucrose in PBS for two additional days. Larvae were keep at −80 °C until they were fixed to a support and coated with Tissue Tech gel (Sakura, Japan). Sections of 30 µm were prepared using the cryostat microtome Leica CM 1510S. Slides with the tissue sections were stored at −20 °C until used (Chakroun and Ferré, 2014).

Histopathological effects were observed in midgut sections after staining with hematoxylin-eosin (Ruiz et al., 2004). Binding of the Vip3Ca protein to the midgut epithelial membrane was performed as described by Chakroun and Ferré (2014) using an anti-Vip3Aa polyclonal antibody coupled to anti-Ig conjugated with FITC.

2.8. Vip3 toxins labeling, preparation of M. brassicae BBMV, and binding analyses

Vip3Aa and Vip3Ca proteins were labeled with biotin using the ECL Protein Biotinylation Module kit (GE Healthcare), according to the manufacturer's instructions. Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method from last instar larvae (Wolfersberger et al., 1987). Protein concentration in BBMV was estimated by the Bradford's method (Bradford, 1976).

For competition experiments, 20 ng of biotinylated toxin was incubated with 20 µg of BBMV in 0.1 ml of binding buffer (PBS pH 7.4, 0.1% BSA) at different concentrations of competitor (unlabeled toxin) at room temperature for 90 min. The reaction was stopped by changing to a new microcentrifuge tube and centrifugation at 16,000*g* for 10 min. The pellet, containing the toxin bound to BBMV, was washed with 500 µl of the binding buffer. The final pellet was resuspended in 10 µl of the binding buffer without BSA and then transferred to a new microcentrifuge tube containing 5 µl of electrophoresis loading buffer (5×). The mixture was heated at 99 °C for 5 min. Samples were run on 10% SDS-PAGE and proteins blotted into a nitrocellulose filter. The filter was blocked with 3 mg/ml of blocking reagent (GE Healtcare) for 1 h. The retained labeled toxins were revealed by probing with streptavidin-conjugated peroxidase (GE Healtcare) and then the Amersham™ ECL™ Western Blotting Detection Reagents luminescence kit.

3. Results

3.1. Host range and insecticidal activity of Vip3Ca

In an attempt to expand the range of susceptible insect species, Vip3Ca was tested with ten new insect species at high protein concentrations (Table 1). Of the six species tested by surface contamination, *C. pomonella* was the most susceptible, followed by *G. mellonella*, with percentages of mortality higher than 50%. From the other types of bioassays, *E. kuehniella* and *T. absoluta* showed some susceptibility to high concentrations of Vip3Ca (between 30% and 50% mortality). The rest of species (*G. molesta*, *P. gossypiella*, *P. xylostella*, *S. nonagrioides*, *D. melanogaster* and *N. viridula*) can be considered non-susceptible, with percentages of mortality lower than 30%.

The LC_{50} of the Vip3Ca protein was calculated for *C. pomonella* (6 µg/cm^2), *E. kuehniella* (180 µg/g), and *M. brassicae* (1.3 µg/cm^2), which was shown to be one of the most susceptible insect species in a previous work (Palma et al., 2012). In addition, the LC_{50} of Vip3Aa to *C. pomonella* was also calculated (0.014 µg/cm^2) for comparison (Table 2).

3.2. Proteolysis of the Vip3Ca protein by midgut juice from different insect species

To determine whether species with different susceptibility to Vip3Ca showed different efficiencies in the activation process of the Vip3Ca protoxin, the time course of the conversion of protoxin (of approximately 90 kDa) into toxin (of approximately 70 kDa) was measured using midgut juice from four lepidopteran species, which, from less to more susceptible to Vip3Ca, were: *O. nubilalis*, *A. ipsilon*, *S. littoralis*, and *M. brassicae* (Palma et al., 2012). The results showed a correlation between susceptibility and speed of protoxin processing (Fig. 1). In the same experimental conditions, the midgut juice of *O. nubilalis* hardly converted the protoxin to the 70 kDa form even at 60 min using the highest concentration of midgut juice. The *A. ipsilon* midgut juice was able to convert

Table 1
Absolute and functional mortality produced by Vip3Ca on ten insect species.

Bioassay method	Insect species	Larval instar	Vip3Ca concentration	Absolute mortality	Functional mortality	Days
Surface contamination	*Cydia pomonella*	Neonate	0.4 μg/cm^2	28.6 ± 0.2	28.6 ± 0.2	7
			4 μg/cm^2	81.20 ± 0.10	100.0 ± 0.0	7
	Grapholita molesta	Neonate	0.4 μg/cm^2	0.10 ± 0.10	0.10 ± 0.10	7
			4 μg/cm^2	22.30 ± 0.10	22.30 ± 0.10	7
	Sesamia nonagrioides	Neonate	0.4 μg/cm^2	6.7 ± 0.7	6.7 ± 0.7	7
			4 μg/cm^2	29.6 ± 0.7	29.6 ± 0.7	7
	Galleria mellonella	L3	0.4 μg/cm^2	36 ± 6	36 ± 6	4
			4 μg/cm^2	54 ± 4	54 ± 4	4
	Plutella xylostella	L3	0.4 μg/cm^2	15.80 ± 0.10	15.80 ± 0.10	7
			4 μg/cm^2	18.80 ± 0.10	18.80 ± 0.10	7
	Drosophila melanogaster	Neonate	0.4 μg/cm^2	0.0 ± 0.0	0.0 ± 0.0	4
			4 μg/cm^2	0.0 ± 0.0	0.0 ± 0.0	4
Diet incorporation	*Pectinophora gossypiella*	Neonate	60 μg/g	9.00 ± 0.10	9.00 ± 0.10	21
	Ephestia kuehniella	L3	100 μg/g	42 ± 13	44 ± 2	4
Leaf disk	*Tuta absoluta*	L2	200 mg/ml	34 ± 10	37 ± 7	3
Feeding assay	*Nezara viridula*	Neonate	1 mg/ml	10 ± 7	10 ± 7	3

Table 2
Quantitative parameters from concentration–mortality responses of some susceptible species to the Vip3Ca protein.

Treatment	Regression line		LC_{50}	Goodness of fit value		95% FL[a]	
	Slope ± SE	a[b] ± SE		χ^2	df[c]	Lower	Upper
E. kuehniella			μg/g				
Vip3Ca[d]	1.6 ± 0.3	1.3 ± 0.6	180	0.55	2	140	270
M. brassicae			μg/cm^2				
Vip3Ca[e]	2.5 ± 0.3	4.70 ± 0.10	1.3	1.83	3	1.1	1.6
Vip3Aa[f]	1.50 ± 0.10	3.30 ± 0.10	0.014	0.9	3	0.011	0.018
C. pomonella			μg/cm^2				
Vip3Ca[e]	0.6 ± 1.0	4.62 ± 0.06	6	4.88	6	3	19
Vip3Aa[e]	0.64 ± 0.12	1.21 ± 0.19	0.013	1.20	2	0.004	0.029

[a] Fiducial limits.
[b] Intercept.
[c] Degrees of freedom.
[d] The LC_{50} was calculated at 3 days.
[e] The LC_{50} was calculated at 7 days.
[f] Data adapted from Ruiz de Escudero et al. (2014).

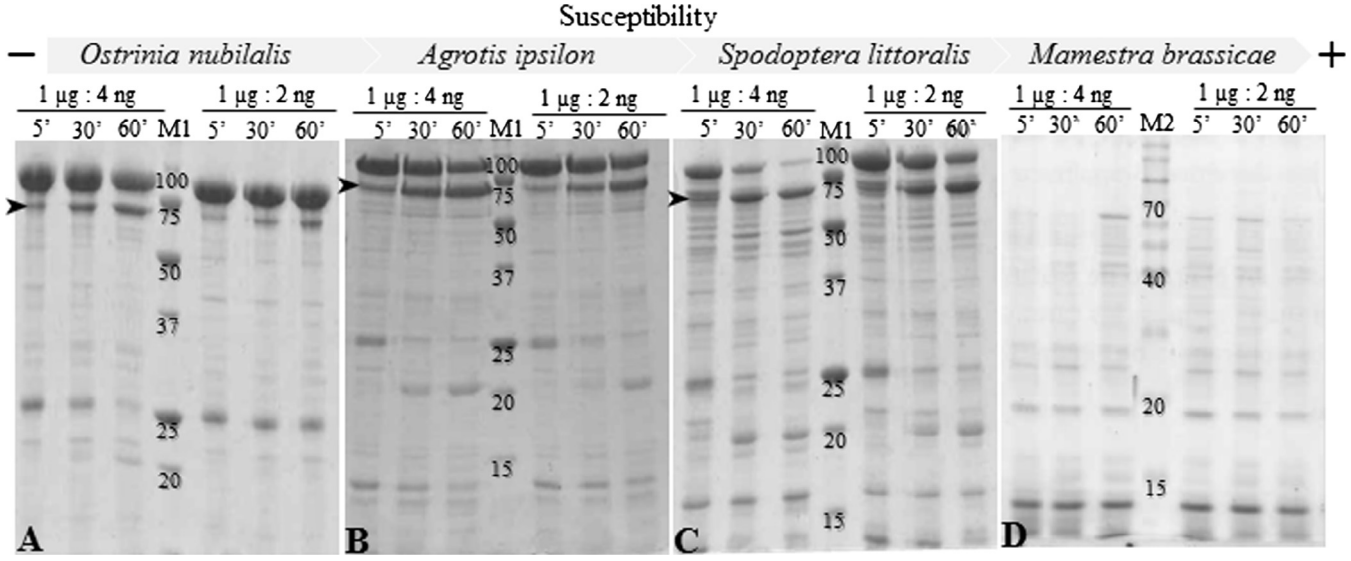

Fig. 1. Time course of the Vip3Ca processing by midgut juice from lepidopteran insects with different susceptibility to the protein. The assay was performed with 10 μg of Vip3Ca in a final volume of 25 μl. Vip3Ca was incubated with 20 ng and 40 ng of total midgut juice protein for 5 min, 30 min and 60 min at 30 °C. Molecular markers (M1 and M2, in kDa) are indicated in the gels. Arrowheads show the position of the activated toxin.

approximately 50% of the protoxin into the 70 kDa form after 60 min. The midgut juice from *S. littoralis* processed most of the protoxin, especially at the highest concentration. The midgut juice of the most susceptible species, *M. brassicae*, not only converted the protoxin to the 70 kDa form but even processed further this form to smaller products.

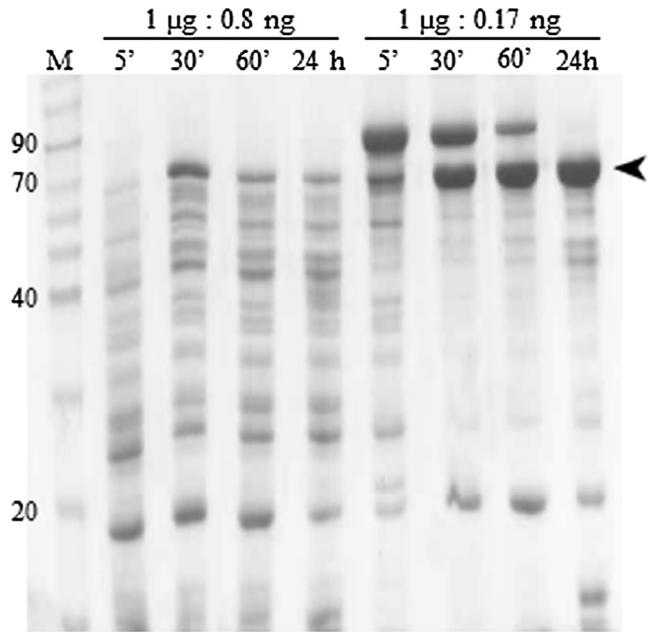

Fig. 2. Time course of the Vip3Ca processing by diluted midgut juice of *M. brassicae*. The assay conditions are as in Fig. 1 except for the ratio Vip3Ca/midgut juice protein and that the assay was extended up to 24 h.

To show that all four species were able to fully convert the protoxin to the activated 70 kDa form given the appropriate conditions, the Vip3Ca protoxin was incubated with different concentrations of midgut juice. Using 20 ng of midgut juice from both *O. nubilalis* and *A. ipsilon* to 1 µg of Vip3Ca, the protoxin was completely converted to the 70 kDa form (data not shown). For *M. brassicae*, the Vip3Ca protoxin was incubated with more diluted midgut juice (0.8 and 0.17 ng of midgut juice total protein for every 1 µg of the Vip3Ca protein). With the lowest dilution, after 24 h the Vip3Ca was completely converted to the 70 kDa form without practically further processing (Fig. 2).

3.3. Insecticidal activity of the Vip3Ca activated with midgut juice of *M. brassicae* and *O. nubilalis*, and with trypsin

To determine whether the low toxicity of Vip3Ca to *O. nubilalis* is related with an inappropriate activation in the larva midgut, *O. nubilalis* neonate larvae were exposed to Vip3Ca activated by midgut juice from *M. brassicae* (a more susceptible species) and, reciprocally, *M. brassicae* neonate larvae were exposed to Vip3Ca activated by midgut juice from *O. nubilalis*. Vip3Aa was used for comparison. The results showed that Vip3Ca activated by midgut juice from *O. nubilalis* was as active, against *M. brassicae*, as the protoxin and the trypsin-activated forms (Fig. 3A). Similar results were obtained for Vip3Aa treated in the same way (Fig. 3B). Similarly, Vip3Ca activated by midgut juice from *M. brassicae* was practically as active against *O. nubilalis* as the trypsin-activated or the non-activated protoxin (Fig. 3A). In the case of Vip3Aa, neither the protoxin nor the activated toxins, by either trypsin or *M. brassicae* midgut juice, were toxic to *O. nubilalis* (Fig. 3B).

3.4. Comparative histopathological effects of Vip3 proteins to *M. brassicae* midgut sections

The histopathological effects of Vip3Aa, Vip3Ca and Vip3Ad to *M. brassicae* were studied in midgut sections of second or third instar larvae which had been fed with a solution of Vip3 protein with sucrose. No tissue damage could be observed after one hour

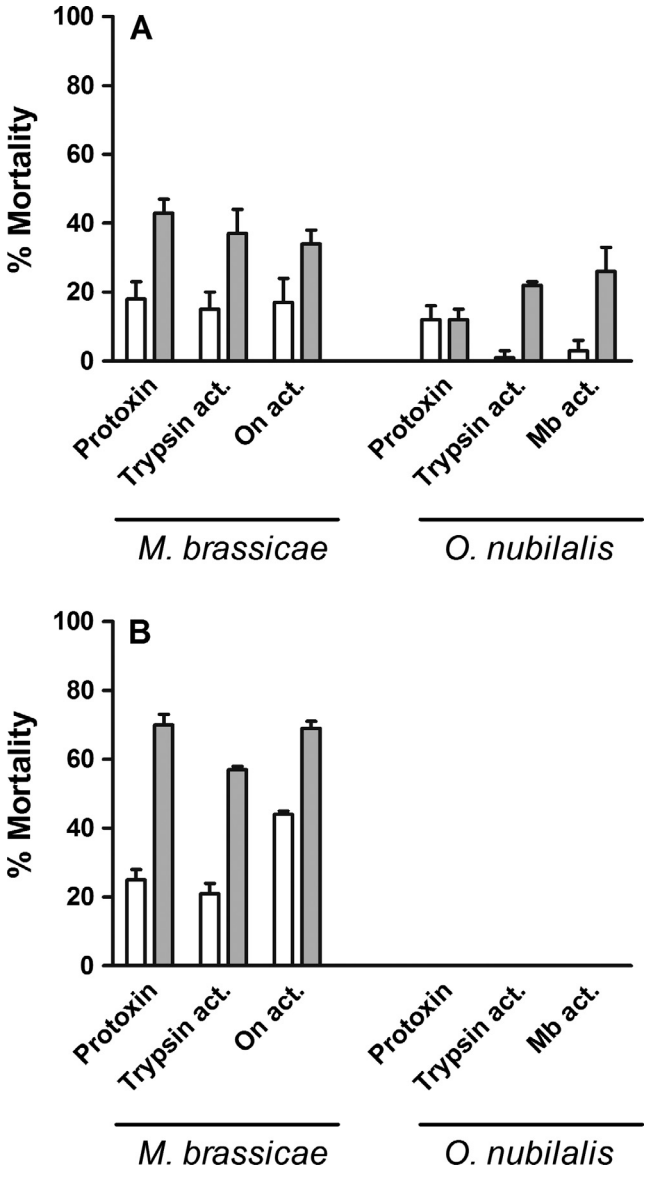

Fig. 3. Toxicity of protoxin, trypsin activated, and midgut juice activated Vip3Ca (A) and Vip3Aa (B) against *M. brassicae* and *O. nubilalis* neonate larvae. Bars represent mean mortality (with SE) of three replicates (of 24 larvae each) after 7 days, at a toxin concentration of 0.74 µg/cm^2 (white bars) and 4.5 µg/cm^2 (grey bars).

of intoxication with any of the Vip3 proteins. After 3 h, visible damage was observed along the whole midgut only in the case of Vip3Aa, the most toxic protein. After 6 h, the midgut was highly damage in larvae that had ingested Vip3Aa. For Vip3Ca, the midgut showed signs of damage but they were not as drastic as for Vip3Aa. For the non-toxic Vip3Ad protein, no damage was evident at any time (Fig. 4).

3.5. In vivo binding of Vip3Ca to *M. brassicae* midgut epithelium

To know if that Vip3Ca binds to the midgut apical membrane after ingestion, larvae were fed with Vip3Ca for different times, dissected and the Vip3Ca protein revealed with a polyclonal Vip3 antibody. Hardly any binding could be detected in larvae that had been dissected one hour after the start of the ingestion. However, after 3 h, binding of Vip3Ca to the brush border membrane was evident (green signal) (Fig. 5). No bound Vip3Ca could be observed after 6 h due to disruption of the apical membrane (as revealed by the loss of the red signal).

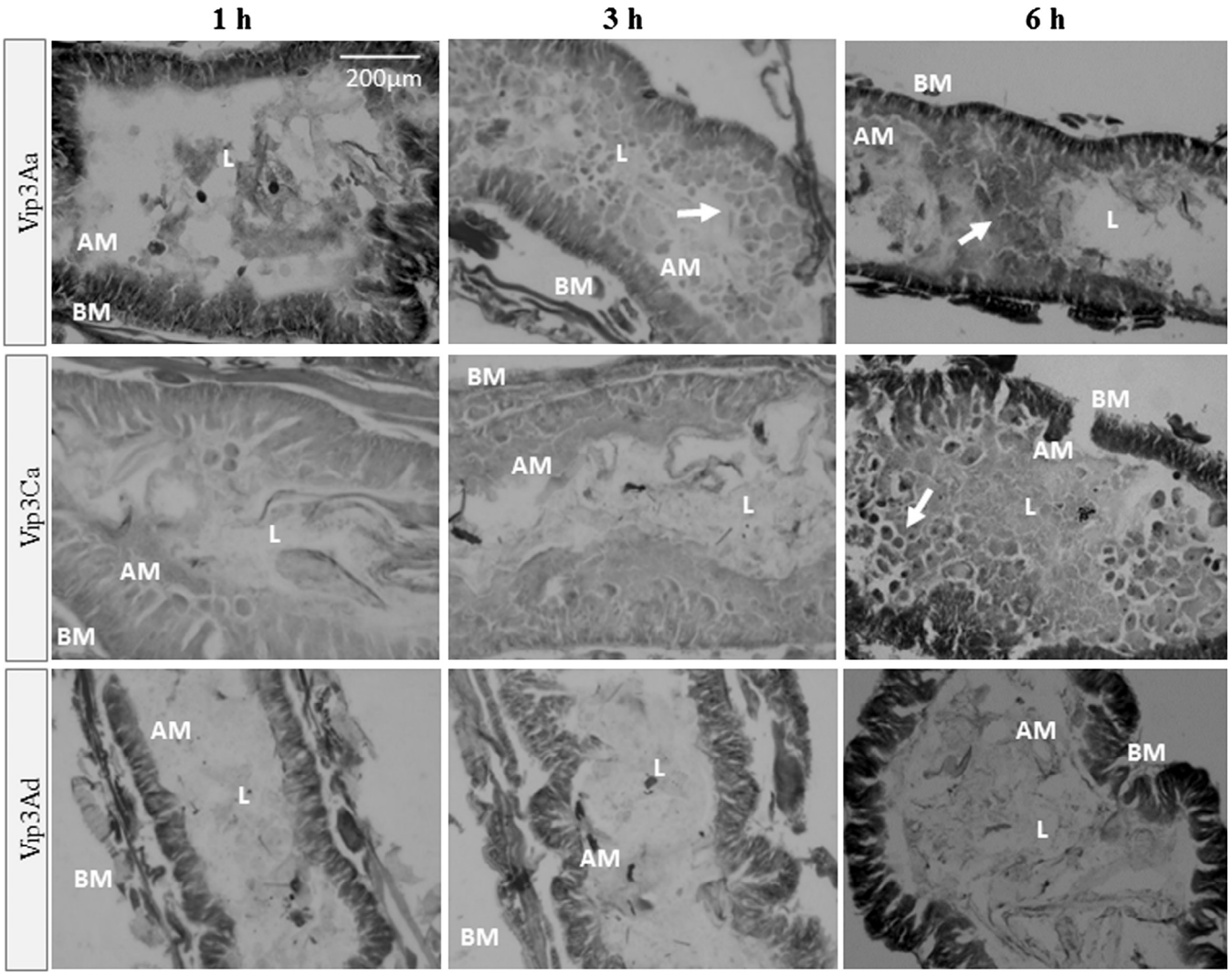

Fig. 4. Histopathological effects of Vip3Ca to the *M. brassicae* midgut after oral ingestion. The Vip3Aa protein was included as a positive control and Vip3Ad protein as a negative control. Midgut sections (30 µm) were stained with hematoxylin-eosin (Ruiz et al., 2004). Magnification 100×. BM, basal membrane; AM, apical membrane; L, gut lumen. White arrows point at excised cells.

3.6. In vitro binding of Vip3 proteins to M. brassicae BBMV

To show that Vip3Aa and Vip3Ca bound specifically to *M. brassicae* BBMV, competition experiments were performed with biotin-labeled toxins. Both toxins bound specifically, since the unlabeled homolog (the same unlabeled toxin as the labeled one) competed for binding with the labeled toxin (Fig. 6A and B). To determine whether the two Vip3 proteins recognized common binding sites, heterologous competition (the competitor is a different toxin than the labeled one) was performed with labeled Vip3Ca and unlabeled Vip3Aa and *vice versa*. The results showed that both toxins competed heterologously with the labeled toxin, indicating that Vip3Aa and Vip3Ca share binding sites (Fig. 6A and C).

4. Discussion

The Vip3Ca protein is the first member of the most recent new family of Vip3 proteins, discovered in a screening program of Spanish collections of *Bacillus thuringiensis*, and it was shown to be toxic to some lepidopteran species, *M. brassicae* among them (Palma et al., 2012). In order to further characterize the insecticidal spectrum of the Vip3Ca protein, we tested ten additional insect species, (eight lepidopterans, one dipteran and one homopteran) (Table 1). LC_{50} values were obtained from the two most susceptible species and also from *E. kuehniella* because of its importance for storage grain in Spain. Compared to Vip3Aa, the LC_{50} values of Vip3Ca for *C. pomonella* and *M. brassicae* were two orders of magnitude higher (Table 2), indicating that Vip3Ca although toxic, is much less toxic than the Vip3Aa protein for these two species.

Since the mode of action of Vip3Ca proteins has never been approached before, we set out to determine the role of proteolysis and binding in the toxicity of this protein. We first studied the processing of the Vip3Ca protein by the midgut juice of *M. brassicae*, *S. littoralis*, *A. ipsilon* and *O. nubilalis* (Figs. 1 and 2). In all cases, the Vip3Ca protoxin was processed to a 70 kDa protein, in contrast to the 62 kDa protein generated from Vip3Aa when incubated with midgut juice (Lee et al., 2003; Chakroun et al., 2012; Caccia et al., 2014). The kinetics of the proteolysis positively correlated with the susceptibility of the species. The conversion of the protoxin into the activated toxin proceeded much faster with the midgut juice of the most susceptible species. This result suggests that the speed of conversion of the protoxin into the 70 kDa toxin might contribute to the susceptibility of the species to the Vip3Ca protein.

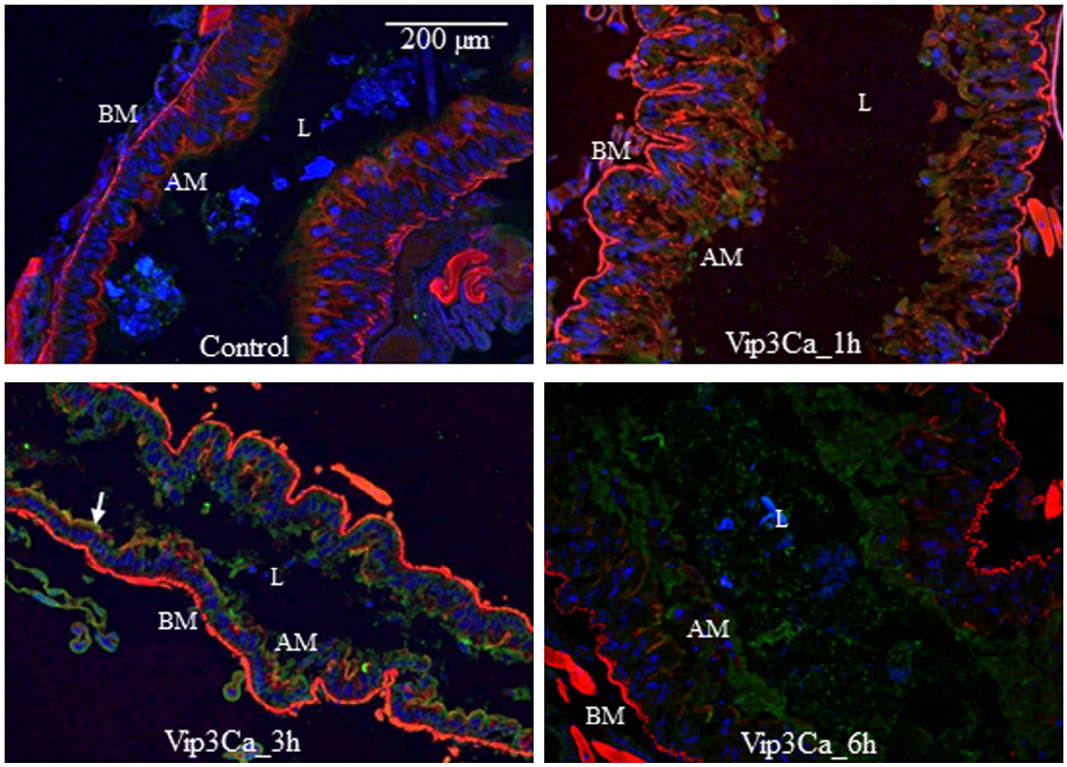

Fig. 5. *In vivo* binding of Vip3Ca in *M. brassicae* after oral ingestion. Larvae fed with just a sucrose solution were used like as negative controls. Binding of the Vip3Ca protein was detected in midgut sections (30 µm) with an anti-Vip3Aa polyclonal antibody and was revealed with anti-IgG coupled with FITC (green fluorescence). Nuclei and actin were stained with DAPI (blue fluorescence) and phalloidin (red fluorescence), respectively. Magnification 100×. BM, basal membrane; AM, apical membrane; L, gut lumen. The white arrow shows binding to the apical membrane.

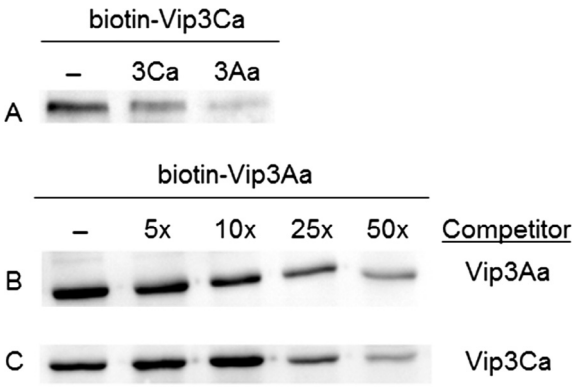

Fig. 6. Binding of biotinylated Vip3Ca and Vip3Aa toxins to *M. brassicae* BBMV. For biotinylated Vip3Ca (A), a 100-fold excess of unlabeled competitor was used (–, no competitor; 3Ca, Vip3Ca; 3Aa, Vip3Aa). For biotinylated Vip3Aa, binding was tested without competitor (–) and in the presence of an excess (5-fold to 50-fold) of unlabeled Vip3Aa (B) and Vip3Ca (C), as indicated on the top of the figure.

In contrast, this type of correlation has not been found for Vip3Aa (Yu et al., 1997).

Yu et al. (1997), showed that the processing of Vip3Aa by midgut juice of different lepidopteran species, including *O. nubilalis*, did not have an effect on its toxicity against them, indicating that midgut processing was not responsible for the very low susceptibility of *O. nubilalis* to this toxin. We have found the same result with Vip3Aa and Vip3Ca using *M. brassicae* and *O. nubilalis* (Fig. 3). Therefore, other factors different to the protoxin activation must be critical in the tolerance of *O. nubilalis* to these two Vip3 toxins.

Several studies have dealt with the histopathological effects of Vip3Aa to larvae from different species (Yu et al., 1997; Abdelkefi-Mesrati et al., 2011a,b; Sellami et al., 2015). We have obtained similar results with *M. brassicae* larvae fed with Vip3Ca, for which disruption of the midgut epithelium could be observed (Fig. 4). However, compared with the most toxic protein Vip3Aa, the damage caused by Vip3Ca took longer to be visible, in agreement with its lower toxicity. Vip3Ad was used as a negative control since it has no toxicity to *M. brassicae* (Ruiz de Escudero et al., 2014). As expected, the Vip3Ad caused no visible damage to the midgut epithelium.

The in vivo binding of Vip3Ca was shown by fluorescence microscopy of the midgut sections stained with specific fluorophores (Fig. 5). The ingested Vip3Ca protein bound to the brush border membrane of the midgut epithelial cells, similarly as it has been reported for Vip3Aa (Yu et al., 1997; Chakroun and Ferré, 2014). To show that binding of Vip3Ca to the brush border membrane is specific, BBMV from *M. brassicae* were prepared and used for *in vitro* binding assays with biotin-labeled Vip3Ca and Vip3Aa. Binding was shown to be specific since homologous competition of Vip3Ca and Vip3Aa was observed (Fig. 6). Heterologous competition experiments also showed that Vip3Aa competed for Vip3Ca binding sites and *vice versa*, indicating that they bind to shared binding sites. Although the overall similarity of the full length Vip3Aa and Vip3Ca proteins is just of 70%, the two proteins share highly conserved regions (92% similarity of the N-terminal region up to amino acid 195 and 66% similarity in a carbohydrate binding domain spanning from amino acid position 544–662) that might be involved in interaction with membrane binding sites. According to our results, at least some of the binding sites would be recognized by both Vip3 proteins. This result might have implications for IRM since, in the event that insects would become resistant to one of these Vip3 proteins due to a change in one of the shared membrane receptors, they could also develop cross-resistance to the other Vip3 protein.

In summary, the Vip3Ca protein (the first representative of this new family of Vip3C proteins) seems to follow a mode of action similar to that of the Vip3Aa protein: it is processed by gut proteases to a smaller molecular weight protein and binds to the brush border of the midgut epithelial membrane, with eventual lysis of midgut cells. Vip3Ca shares binding sites with Vip3Aa, which might have implications for IRM. It seems that there is a difference compared to Vip3Aa mode of action, since the kinetics of the activation of Vip3Ca may play a role in the degree of susceptibility of susceptible insects. With this study, we aimed to shed some light on the activity spectrum and mode of action of the recent new family of Vip3C proteins.

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